Equilibrium and pre-equilibrium fluorescence spectroscopic studies of the binding of a single-immunoglobulin-binding domain derived from Protein G to the Fc fragment from human IgG_1

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A single-immunoglobulin-binding protein based upon the C2 domain of Protein G from Streptococcus has been shown to bind tightly to the Fc fragment of IgG_1. The binding interaction results in a decrease in the fluorescence intensity from the sole Trp residue (Trp-48) in this domain. This spectral change has been used to monitor the binding interactions between the two proteins using equilibrium and pre-equilibrium fluorescence spectroscopy. Comparison of the data from the two techniques suggests that a conformational change occurs after the initial formation of the complex. Mutagenesis studies have shown that the Trp residue is important for binding and that replacement by a Phe residue leads to a 300-fold decrease in the affinity for $Fc_{\gamma 1}$. Determination of the rate constants k_{on} and k_{off} at different values of pH between 4.0 and 9.0 suggest that variations in K_d are mediated predominantly by changes in k_{on}. Competition experiments between SpG[1] and a single-IgG-binding domain from Protein A from *Staphylococcus aureus* have been used to determine the affinity of the latter for $Fc_{\gamma 1}$.

INTRODUCTION

Many Staphylococcal and Streptococcal strains have multi-domain proteins on their cell surface which bind to an array of serum proteins, such as immunoglobulins, albumin and blood clotting factors [1]. The biological role of these cell surface proteins is not known; however, it has been proposed that by binding host proteins on their surface the bacteria are able to evade attack from the host's immune system [2]. The IgG-binding activity of Protein A from *Staphylococcus aureus* has been characterized in great detail and has been widely used in a variety of immunological techniques [3]. Protein G from the Streptococcal groups G and C binds to a wider spectrum of IgG subclasses than Protein A [4], thus demonstrating that its binding interactions are not identical to those of Protein A. Both Protein A and Protein G show high affinity for the C_H2–C_H3 interface region of IgG [5]. Protein G also binds to the C_H1 domain of the Fab fragment [6–8]. Due to the high affinity and complementary specificity with which SpG (a single-IgG-binding domain from Protein G) interacts with IgG, this protein has also found use in many immunological applications.

The genes encoding Protein G from a number of Streptococcal strains [9–11] have been sequenced and have revealed a multi-domain protein with separate regions capable of binding IgG and albumin [12]. There may be two or three highly homologous IgG-binding domains (55 amino acid residues in length) in Protein G depending upon the strain from which the protein is isolated. These domains are separated by an extended 15-amino-acid spacer region. Despite the similarities between Protein A and Protein G with respect to their mode of action, the gene sequences and amino acid sequences show no similarity. The IgG-binding domains of Protein G have been expressed in *Escherichia coli* as single- or multiple-domain proteins which show a high affinity for IgG. Both NMR [13–17] and X-ray crystallography [18,19] have been performed on these individual IgG-binding domains. These studies revealed that the global fold of the protein consists of a four-stranded β sheet spanned by a single helix lying diagonally across the β sheet. The two central β strands of the sheet are parallel and contain both the N- and C-terminal residues (Figure 1). This arrangement appears to make the IgG-binding domains particularly stable, the B1 and B2 domains have melting temperatures of 87.5 °C and 79.4 °C respectively at pH 5.4 [20].

It is noteworthy that each of the IgG-binding domains of Protein A, which bind to the same region of the Fc fragment, is unrelated in sequence to those of Protein G and is also unrelated in structure, being composed of a tri-helical bundle [21]. The residues involved in IgG binding are located within the first two α-helices [22]. Chemical shift perturbation experiments found that the site of interaction between the B1 domain of Protein G and the human Fc fragment is formed by parts of β strand 3 and the α-helix [23]. This agrees with peptide inhibition studies which also found the same region to be crucial to the interaction [24]. Involvement of the α-helix has also been implicated by hydrogen–deuterium exchange protection experiments [16]. The X-ray and NMR structures of a complex formed between an IgG-binding domain from Protein G and mouse Fab have been reported [6,7]. These structural determinations show that the interaction occurs between β strand 2 of Protein G and the last β strand of the C_H1 domain. It appears, therefore, that the interaction with Fab involves different regions of the IgG-binding domain than those used in the interaction with Fc.

Lancet and co-workers [25] demonstrated that a quenching of the fluorescence at 330 nm occurred from a solution containing the Fc fragment from rabbit IgG when aliquots of a solution of the B domain of Protein A were added to it. Since the latter

Abbreviations used: SpG[1], single-immunoglobulin-binding protein based upon the C2 domain of Protein G from Streptococcus; SpG, single-IgG-binding domain from Protein G; SpA_B*-1 and SpA_B*-2, recombinant proteins with one or two consecutive IgG-binding domains respectively, each based upon the B domain of Protein A from *Staphylococcus aureus*; $Fc_{\gamma 1}$, Fc fragment from IgG_1; IgG–HRP, rabbit anti-mouse IgG complexed with horseradish peroxidase.

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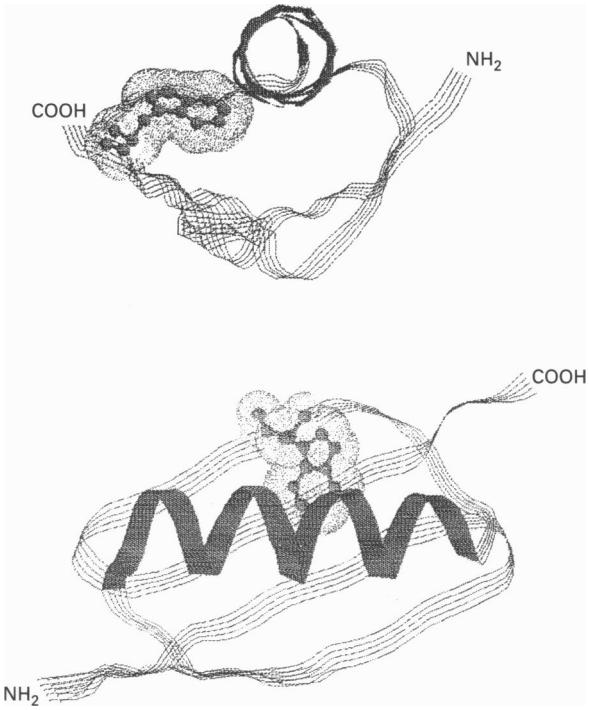

Figure 1 Ribbon diagram of SpG¹

Ribbon diagrams showing two orthogonal views of SpG¹; the secondary structural elements and the partially buried position of Trp-48 are shown. The figure was generated using Rasmol 2.5 (R. Sayle, Glaxo Research and Development, Greenford, Middlesex, U.K.; 1994)

contains no Trp residues, the quenching phenomenon was assigned to Trp residues in the Fc molecule. It has been proposed that Protein G competes for the same site on the IgG molecule as Protein A [5], and it was therefore considered possible that the binding of Protein G to IgG may also lead to a change in the fluorescence properties originating either from Trp residues in the rabbit Fc or from Protein G itself, since this contains one Trp residue per IgG-binding domain (see Figure 1).

In order to investigate the interaction between Protein G and IgG further, a synthetic gene which coded for an individual IgG-binding domain based upon the C2 domain of Protein G (from strain G148 [26]) was made (designated SpG¹). An additional six residues (residues MTPAVS) were incorporated at the N-terminus to facilitate cloning and expression and a cysteine residue was added at the C-terminus to facilitate immobilization on to Sepharose for other studies [27]. This construct has allowed detailed analysis of the SpG¹–$Fc_{\gamma 1}$ interaction (where $Fc_{\gamma 1}$ is the Fc Fragment from IgG_1) through site-directed mutagenesis techniques [27] and the use of the fluorescence spectroscopic studies reported here.

MATERIALS AND METHODS

Chemicals were obtained from Sigma Chemical Co., Poole, Dorset, U.K., or BDH, Poole, Dorset, U.K. Rabbit anti-mouse IgG–horseradish peroxidase (HRP) was purchased from Serotec Ltd., Oxford, U.K. Human $Fc_{\gamma 1}$ was a gift from Dr. M. Glennie, Tenovus Laboratories, Southampton, U.K.

Preparation of SpG¹

The construction of the gene encoding SpG¹, its cloning, expression and the purification of the protein have all been described elsewhere [27] and full details are available from the corresponding author (M.G.G.).

Mutagenesis

Site-directed mutagenesis was performed using the mismatch primer method [28]. The oligonucleotide was synthesized on an Applied Biosystems 381A DNA synthesizer. The mismatch primer oligonucleotide sequence used for the W48F mutation was 5′-GTC GTC GTA GGT GAA TTC ACC GTC GA-3′. The mutated gene was verified by DNA sequencing. The mutated protein was expressed and purified in a similar manner to SpG¹.

Estimation of protein concentration

Protein concentrations were estimated by a bicinchoninic acid assay [29]. Chemicals were obtained from Sigma and used according to their instructions.

Competitive ELISA

The wells of a 96-well microtitre plate were coated with 200 μl of coating buffer (0.8 g of Na_2CO_3, 1.46 g of $NaHCO_3$ per 500 ml, pH 9.0) containing 120 ng of a two-domain IgG-binding protein based upon the B domain of Protein A, called SpA_B*-2 (for a complete description see [30]). After 2 h at 37 °C (or overnight at room temperature) the plate was washed with PBST (PBS/0.01 % Tween 20, pH 6.0) three times. Then 100 μl of PBST was added to each well, 50 μl of competing protein solution in PBST was added to lane 2 and serially diluted (3-fold) across the plate leaving the appropriate lanes blank as controls, and then 100 μl of IgG–HRP previously diluted 1:1250 in PBST was added to all the wells. The plate was left for 30 min at room temperature before washing and developing as described in [30].

Fluorescence measurements

All fluorescence measurements were obtained using an Hitachi F2000 spectrofluorimeter with the temperature maintained at 25 °C by a thermally jacketed cuvette holder. Scans were recorded using an excitation and emission slit-width of 5 nm and at a speed of 15 nm/min using an excitation wavelength of 286 nm. All measurements were corrected, where necessary, for the inner filter effect [31].

Fluorescence titrations

All titrations were carried out at 25 °C, using 1 ml of 100 nM human $Fc_{\gamma 1}$ and aliquots of 250 μM SpG¹. Unless otherwise specified, the buffer used was 20 mM phosphate, pH 6.0. The excitation wavelength was 286 nm and the fluorescence emission was detected at 336 nm. Readings were averaged over a 10 s period with a 10 s delay to allow for equilibration. Each titration and calculation was repeated three times and all values given are mean values for the three experiments with S.D.s given in parentheses.

Stopped-flow measurements

All stopped-flow measurements were made on an Applied Photophysics SX.17MV stopped-flow spectrofluorimeter fitted with 2 ml syringes. The temperature was maintained at 25 °C, unless otherwise stated, with a Neslab RTE-111 water bath. All measurements were taken over a 0.5 s time scan with 2000 data

points. An excitation wavelength of 280 nm was selected by a monochrometer and the fluorescence emission above 335 nm was measured using a suitable glass filter. All of the solutions of SpG[1] and human $Fc_{\gamma 1}$ were prepared in 20 mM potassium phosphate buffer at a selected pH. Both solutions were filtered prior to use to minimize light scattering interference and were thermo-equilibrated at the correct temperature prior to use. The reaction progress curves generated were analysed by the manufacturer's software using a single- or double-exponential curve-fitting algorithm.

The experiments to determine the rate constant k_{off} by direct observation of the dissociation of the pre-formed complex Fc·SpG[1] were carried out as follows. A solution of complex formed by the addition of 20 μM SpG[1] to 10 μM Fc was rapidly diluted 10:1 (v/v) with buffer containing various concentrations of SpG[1] at 13 °C. The resultant increase in fluorescence intensity was analysed as above.

RESULTS

The role of Trp-48

We have previously cloned and expressed a single-IgG-binding domain, SpG[1], based upon the C2 domain of Protein G [27]. In this paper we report the results of experiments in which SpG[1] was used in fluorescence spectroscopic studies to investigate the events involved in the Protein G–$Fc_{\gamma 1}$ interaction. SpG[1] possesses a unique Trp residue at position 48. This is equivalent to residue 43 in the single-Ig-binding protein used by Fahnestock and co-workers [10], and the involvement of this residue in the SpG–Fc interaction has been illustrated in the chemical shift perturbation studies of Gronenborn and Marius Clore [23] and in peptide mapping studies [24]. These observations have been supported by mutagenesis studies on our construct in which Trp-48 was replaced by Phe-48 and the effect of the mutation was determined by competitive ELISA experiments. Figure 2 shows that the concentration of the mutant required to inhibit the binding of rabbit anti-mouse IgG–HRP is considerably higher (greater than 300-fold) than the required concentration of the wild-type SpG[1],

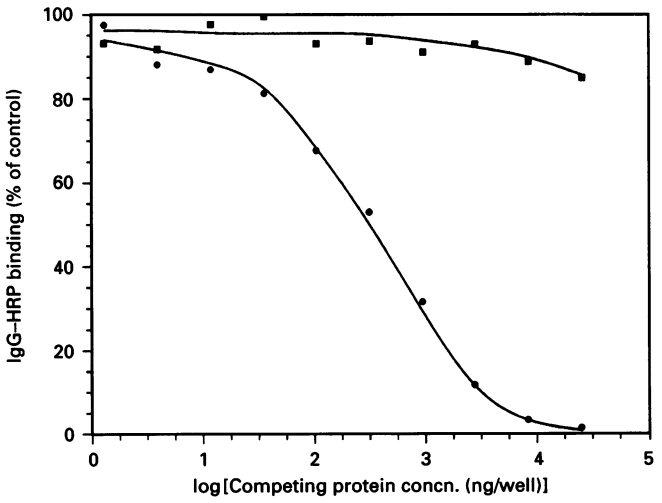

Figure 2 Competitive ELISA showing the effect of the W48F replacement

ELISA curves describing the competition between native (●) or W48F mutated (■) SpG[1] and immobilized SpA_B*-2 for the rabbit anti-mouse IgG–HRP complex. The protocol is fully described in the Materials and methods section.

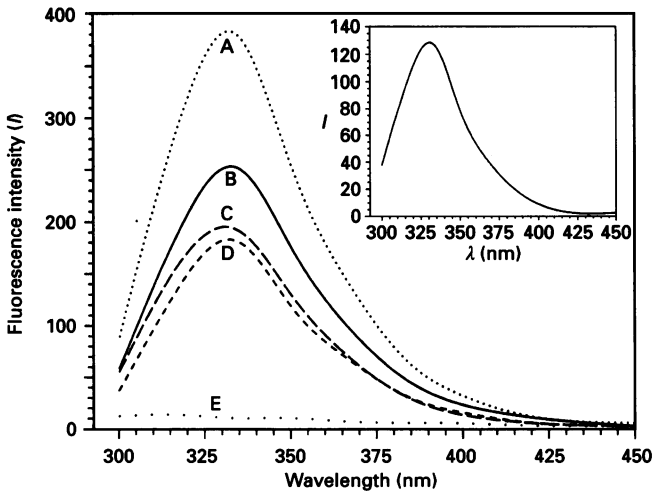

Figure 3 Fluorescence emission spectra of native SpG[1] in free solution or mixed with $Fc_{\gamma 1}$

Solutions of 200 nM SpG[1] (curve D) or 200 nM $Fc_{\gamma 1}$ (C) or a mixture of the two (B) were excited by light at 286 nm and the fluorescence emission spectra determined. The emission spectrum of 200 nM SpG[1]-W48F (E) determined under similar conditions is also shown. Curve A is the theoretical spectrum obtained by numerical summation of the spectra for free SpG[1] and $Fc_{\gamma 1}$. The inset shows the difference spectrum between the theoretical and experimentally determined spectra for the complex formed between SpG[1] and $Fc_{\gamma 1}$. The buffer used in all cases was 20 mM potassium phosphate, pH 6.0, at 25 °C.

showing that this mutation causes a dramatic reduction in the affinity of the protein for IgG–HRP. The effect of replacing Trp-48 with a Phe can also be seen in a comparison of the fluorescence emission spectra of SpG[1] and SpG[1]-W48F, which are shown in Figure 3. SpG[1] displays an emission maximum at 338 nm, indicative of a partially exposed Trp residue (see Figure 1). In contrast, the emission spectrum of SpG[1]-W48F shows no characteristic Trp fluorescence (as would be expected) but shows some weak fluorescence emission at wavelengths around 310 nm (Figure 3), typical of that from Tyr residues [32].

Preliminary experiments using the fluorescence properties of SpG[1] showed that the summated fluorescence intensities at 336 nm from solutions of IgG (200 nM) and SpG[1] (200 nM) was greater than that measured when the same concentrations of each protein were mixed together. This observation suggested that the formation of a complex between the two proteins may be monitored fluorimetrically. SpG has been demonstrated to interact with human IgG via both the Fc and the Fab fragments [5]. Therefore the possibility of fluorescence signal changes arising from the latter interactions was eliminated by using only $Fc_{\gamma 1}$ rather than IgG_1. The emission spectra for 200 nM SpG[1], 200 nM $Fc_{\gamma 1}$ and a mixture of the two in 20 mM potassium phosphate buffer, pH 6.0, at 25 °C are shown in Figure 3. This experiment showed that the interaction between SpG[1] and $Fc_{\gamma 1}$ results in a decrease in fluorescence intensity. As is evident from these spectra, the fluorescence intensity observed from the mixture is significantly less (35%) than the sum of the intensities of the two separate components. The quenching of fluorescence emission that occurs on complex formation is represented as a fluorescence difference spectrum in the inset to Figure 3.

Determination of the K_d for the binding equilibrium

In order to use the change in fluorescence properties of the protein complex to monitor the binding interactions, 1 ml

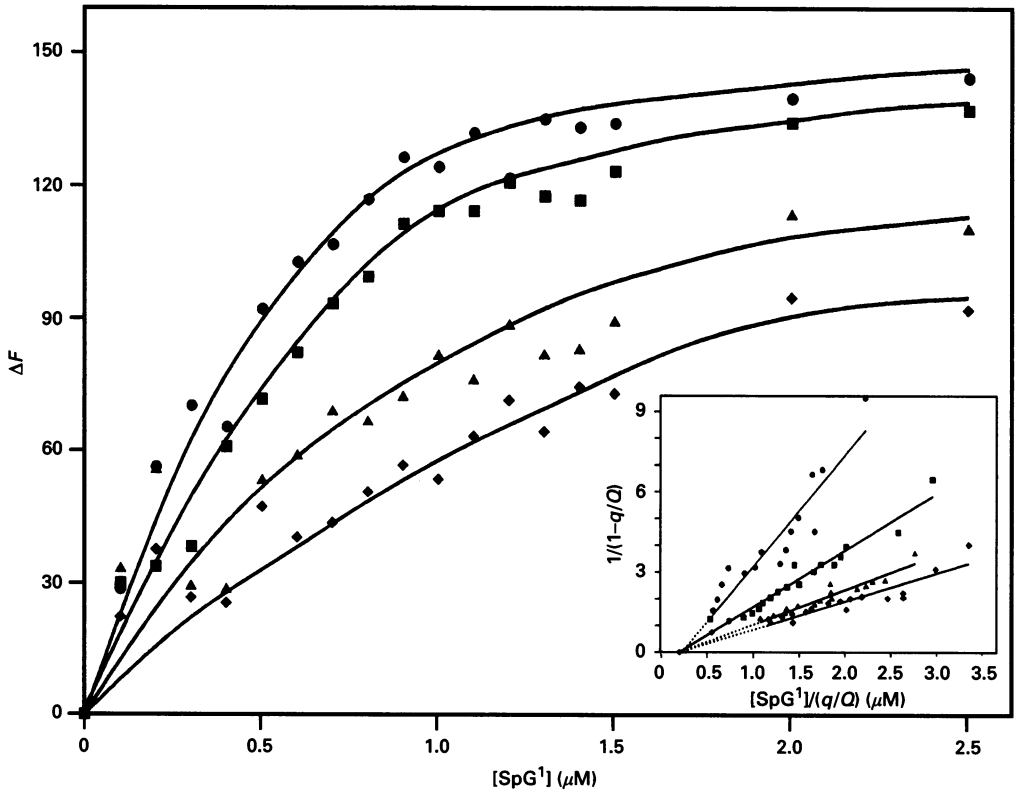

Figure 4 Effect of pH on the saturation of $Fc_{\gamma 1}$ by SpG^1

SpG^1 was titrated into solutions of 100 nM $Fc_{\gamma 1}$ in phosphate buffer at pH 6.0 (●), 7.0 (■), 8.0 (▲) and 9.0 (◆) and the fluorescence emission at 336 nm was monitored. Similar titrations into buffer alone at each pH value were also carried out. The latter curves together with the initial fluorescence intensity at 336 nm of the $Fc_{\gamma 1}$ alone were subtracted from the test titrations to generate the saturation curves shown. The inset shows the analyses of the saturation curves by the Klotz analysis.

samples of $Fc_{\gamma 1}$ (0.1 μM) were titrated by addition of a solution of SpG^1 and the fluorescence emission was monitored at 336 nm. The change in fluorescence intensity was also measured when SpG^1 was added to buffer alone. The amount of fluorescence quenching occurring at each concentration of SpG^1 (calculated by the subtraction of the fluorescence intensity of a solution of the mixed proteins from the summated fluorescence intensities of the two individual proteins) was then plotted against the concentration of SpG^1 to give a saturation curve (see Figure 4 and inset). Each titration was carried out in triplicate.

The dissociation constant determined at pH 6.0, where SpG^1 is most active, was 306 (±65) nM. This is indicative of very tight binding and is in good agreement with the value of 333 nM for the B1 domain (≡C1) obtained by Gallagher et al. [19] using titration calorimetry. Approximately 2 mol equivalents of SpG^1 were shown to bind to 1 mol of $Fc_{\gamma 1}$, as might be expected since $Fc_{\gamma 1}$ has two heavy chains, each with a potential binding site for SpG^1 at the C_H2–C_H3 interface.

Fluorescence studies using the mutant W48F

When a mutant bearing a W48F replacement was used in the titration experiments no change in the fluorescence intensity of the mixture occurred, suggesting either that the binding was very weak or that no change in fluorescence occurs when this mutant binds to the $Fc_{\gamma 1}$. However, ELISA data (see Figure 2) show that the W48F mutant protein has a much lower affinity (minimum K_d = 90 μM) for $Fc_{\gamma 1}$ than the non-mutated protein (K_d = 310 nM), emphasizing the important role played by Trp-48 in the binding interactions. This loss of affinity caused by the W48F substitution occurs without significant changes in the structure of the protein, as determined by CD spectroscopy [27].

The effect of pH on K_d

In order to confirm that the interaction reported by the change in fluorescence intensity is that associated with formation of the complex between SpG^1 and $Fc_{\gamma 1}$, and to probe the electrostatic nature of the interaction between SpG^1 and human $Fc_{\gamma 1}$, the effect of pH on the dissociation constant was measured. Titrations were carried out at pH 4, 5, 6, 7, 8 and 9. The effect of pH on the binding between $Fc_{\gamma 1}$ and SpG^1 and the corresponding K_d values can be seen in Figure 4 and Table 1. These results show that the pH optimum for the binding interaction is around pH 6.0, in close agreement with results found by immobilization techniques [33] for Protein G.

Determination of the K_d for the interaction between $Fc_{\gamma 1}$ and the B domain of Protein A

The interaction between the B domain of Protein A and human Fc does not produce a measurable change in fluorescence intensity [25]. However, since the B domain has been shown to competitively inhibit the interaction of the IgG-binding domains of

Table 1 Effect of pH on the binding of SpG^1 to $Fc_{\gamma 1}$

The effect of pH was measured on the number of equivalents (n) of SpG^1 bound to $Fc_{\gamma 1}$ and on the value of the dissociation constant (K_d) for the equilibrium between $Fc_{\gamma 1}$, SpG^1 and the complex $Fc_{\gamma 1}$·SpG^1 determined at equilibrium. Also shown are the rates k_{on} and k_{off} and the K_d calculated from the latter two rate constants. Values in parentheses are S.D.s. nd = not determined.

	Equilibrium		Pre-equilibrium		
pH	K_d (nM)	n	$10^6 \times k_{on}$ ($M^{-1} \cdot s^{-1}$)	k_{off} (s^{-1})	K_d (μM)
4.0	560 (82)	1.7 (0.5)	0.138	1.017	7.369
5.0	398 (56)	1.9 (0.1)	0.661	1.468	2.221
6.0	306 (65)	2.2 (0.1)	0.621	1.245	2.005
7.0	459 (32)	2.1 (0.1)	0.358	0.965	2.696
8.0	693 (100)	1.9 (0.1)	0.142	0.758	5.338
9.0	928 (51)	1.9 (0.1)	nd	nd	nd

Protein G with Fc [5], it is possible to determine the K_d for its binding interaction with $Fc_{\gamma 1}$ indirectly by its competitive inhibition of SpG^1 binding in fluorescence titration experiments. By measuring the apparent K_d ($K_{d,app.}$) of SpG^1 in the presence of a known concentration of Protein A, the dissociation constant at equilibrium for the latter can be calculated.

Fluorescence titrations were carried out as described above for SpG^1 in the presence of different concentrations of SpA_B*-1, a single-IgG-binding domain based on domain B of Protein A [30].

Saturation curves obtained from individual titrations can be seen in Figure 5 and show quite clearly the reduction in the apparent affinity of SpG^1 for $Fc_{\gamma 1}$ that would be expected in the presence of a competitive inhibitor. These experiments suggested a value of 22.5 (±4.6) nM for the K_d of the equilibrium between $Fc_{\gamma 1}$ and SpA_B*-1, which is lower than the K_d of 40 nM obtained by ELISA for this protein and that obtained for $Fc_{\gamma 1}$–SpG^1 binding.

Observation of the formation of the SpG^1·$Fc_{\gamma 1}$ complex by stopped-flow fluorescence

In the formation of the SpG^1·$Fc_{\gamma 1}$ complex, the K_d is described by the ratio of the rates of association (k_{on}) and dissociation (k_{off}) by the relationship $K_d = k_{off}/k_{on}$. Using the observed fluorescence change which accompanies SpG^1–$Fc_{\gamma 1}$ binding, these two rate constants can be determined by use of a stopped-flow spectrofluorimeter by monitoring the change of fluorescence intensity after rapid mixing of SpG^1 and $Fc_{\gamma 1}$.

Determination of k_{on}

For the experiments to determine k_{on} the concentration of $Fc_{\gamma 1}$ was fixed at 1 μM (final concentration after mixing had occurred) and the concentration of SpG^1 was varied between 1.87 and 22 μM (final concentrations). Typical reaction progress curves for different concentrations of SpG^1 are shown in Figure 6. The reaction progress curve shown in Figure 6 fits a single-exponential

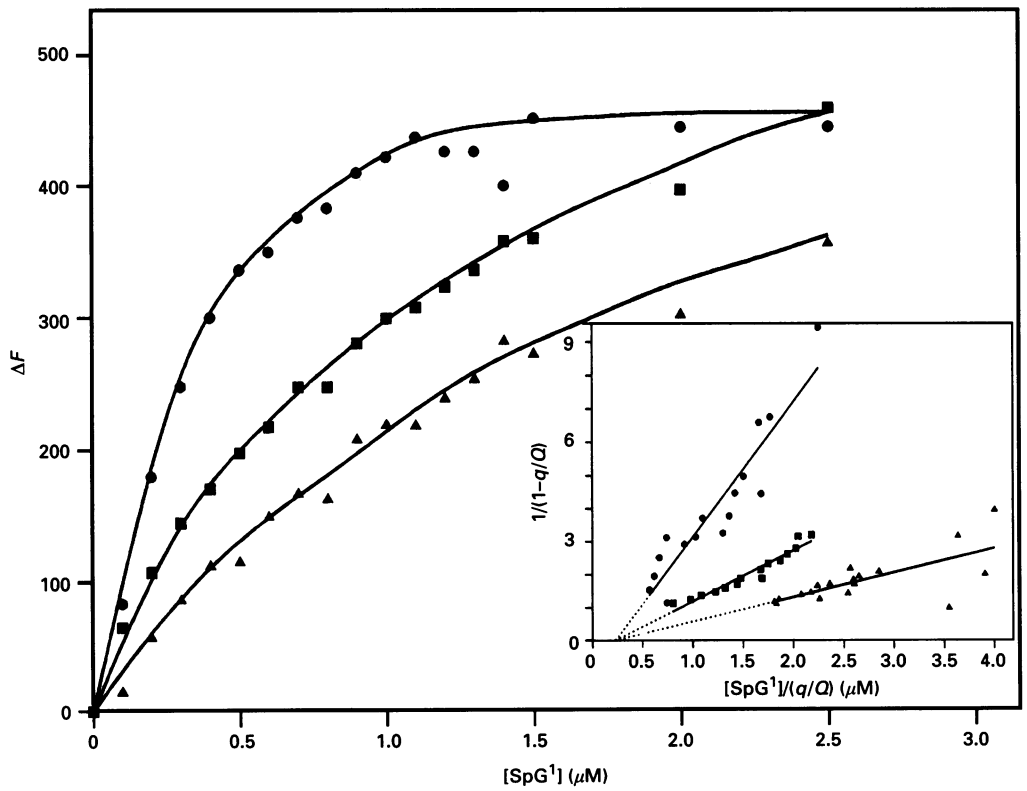

Figure 5 Titration curves showing the saturation of $Fc_{\gamma 1}$ by SpG^1 in the presence of different concentrations of SpA_B*-1

Titrations were carried out as described in the legend to Figure 4 except that the SpG^1 was added to $Fc_{\gamma 1}$ in the presence of 0 (●), 30 nM (■) or 60 nM (▲) SpA_B*-1. The inset shows the corresponding Klotz analysis.

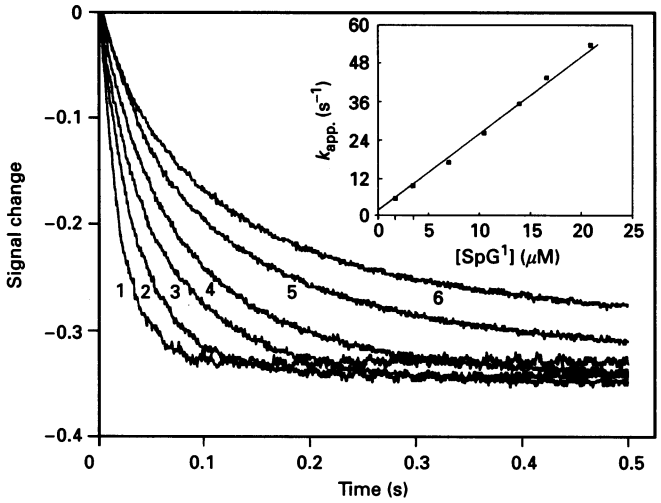

Figure 6 Stopped-flow fluorescence analysis of the formation of the $Fc_{\gamma 1}$·SpG^1 complex

Equal volumes of $Fc_{\gamma 1}$ (2.0 μM; final concentration 1.0 μM) and various concentrations of SpG^1 were mixed in 20 mM potassium phosphate buffer (25 °C) and any fluorescence changes that occurred were followed. The excitation wavelength used was 280 nm and the emission wavelength at 335 nm was selected by a filter. The traces shown were obtained using the following final concentrations of SpG^1: 1, 22 μM; 2, 18 μM; 3, 14 μM; 4, 11 μM; 5, 9 μM; 6, 7 μM. The inset is the graphical determination [37] of the rate constants k_{on} and k_{off} from the pseudo-first-order rates ($k_{app.}$) calculated for each curve. k_{on} is given by the slope of the line and is equal to 1.9×10^{-6} $M^{-1} \cdot s^{-1}$. k_{off} is given by the intercept on the y axis and is equal to 3.0 s^{-1}.

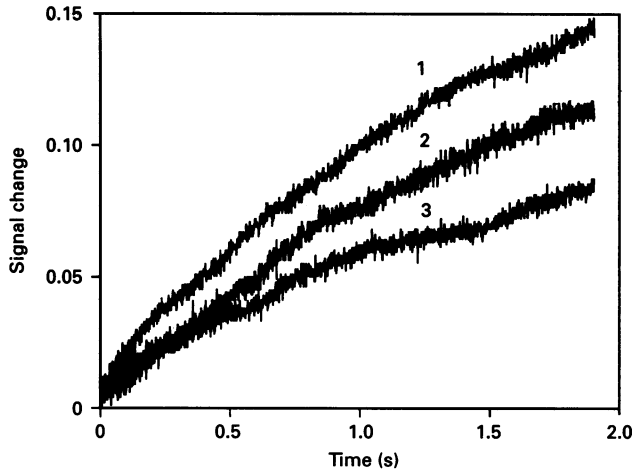

Figure 7 Dissociation of the $Fc_{\gamma 1}$·SpG^1 complex

A solution of $Fc_{\gamma 1}$·SpG^1 complex (10 μM $Fc_{\gamma 1}$ + 10 μM SpG^1) was rapidly mixed with a 10-fold (v/v) excess of buffer (20 mM potassium phosphate buffer, pH 8.0, at 15 °C) and the resultant change in fluorescence intensity at 335 nm monitored. Traces 1, 2 and 3 were generated by dilution of the above complex by buffer alone, or buffer containing 0.2 μM SpG^1 or 0.4 μM SpG^1 respectively.

equation, suggesting that the reaction occurs by a simple process. The data given in Figure 6 show that the increase in $k_{app.}$ is linearly proportional to an increase in concentration of SpG^1, demonstrating that at these levels of reactant the rate-limiting step is the formation of the binary complex SpG^1·$Fc_{\gamma 1}$. The value of k_{on} derived from these data is 1.9 $\mu M^{-1} \cdot s^{-1}$.

Determination of k_{off}

From the plot shown in the inset to Figure 6 it is also possible to estimate k_{off} from the intercept on the y axis to be approx. 3.0 ± 1 s^{-1}. The two values result in an estimated K_d of 1.6 μM at pH 6.0. This is approx. 5–6-fold higher than that determined from studies using fluorescence spectroscopy at equilibrium.

Effect of the concentration of free SpG^1 on k_{off}

The rate of dissociation of the complex can also be determined directly by rapid dilution of a pre-formed complex of $Fc_{\gamma 1}$·SpG^1 and monitoring the increase in fluorescence intensity. Experiments were carried out in which pre-formed complexes of $Fc_{\gamma 1}$·SpG^1 at pH 6.0 were diluted 10-fold by buffer at pH 8.0 (final pH 7.9, to maximize the amount of dissociation achieved) containing various concentrations of SpG^1. Figure 7 shows typical increases in fluorescence intensity at 335 nm when a solution of 10 μM $Fc_{\gamma 1}$ and 20 μM SpG^1 was rapidly diluted 10:1 (v/v) into 20 mM potassium phosphate buffer, pH 8.0, containing 0, 0.2 μM or 0.4 μM SpG^1. The single-exponential analyses of the reaction progress curves show that at 13.0 °C the rates of dissociation were approximately the same (0.84 s^{-1}, 0.85 s^{-1} and 0.85 s^{-1} respectively) and therefore independent of the concentration of free SpG^1 In contrast, however, the amplitudes of the fluorescence changes noted on dilution were 0.24, 0.22 and 0.16 respectively, indicating that the position of the equilibrium attained was dependent on the concentration of SpG^1 used. These two observations suggest that the dissociation of SpG^1 from the complex is rate-limited by another process such as a preceding structural change in the complex.

Effect of SpA_B*-1 on the binding of SpG^1 to $Fc_{\gamma 1}$

Additional experiments were carried out in the presence of a 10-fold molar excess of SpA_B*-1 pre-mixed with the $Fc_{\gamma 1}$. Under these conditions no change in fluorescence was observed upon rapid addition of SpG^1. This would be expected from the results obtained above which demonstrated that SpA_B*-1 is a competitive inhibitor to SpG^1 binding.

Effect of pH on k_{on} and k_{off}

The binding affinity of SpG^1 for $Fc_{\gamma 1}$ shows a maximum at pH values around 6.0 (see above). The different affinities at pH values outside this range may be caused by a change in k_{on}, k_{off} or a combination of both. Experiments, as described above, were therefore carried out to investigate these possibilities. Both proteins were dissolved in the same buffer at a pH value selected between 4.0 and 8.0. Although 20 mM phosphate buffer does not buffer well over this range it was shown that the pH of each solution would not change during the formation of the complex at the protein concentrations used. These experiments were performed at 13.0 °C to minimize protein instability at the more extreme pH conditions.

Table 1 shows the values of k_{on}, k_{off} and the calculated value of K_d for the reactions carried out at pH 4.0 to pH 8.0. The calculated K_d values show that SpG^1 has its highest affinity for $Fc_{\gamma 1}$ at pH 6.0, in good agreement with experiments performed at equilibrium. The effect of pH on K_d appears to be primarily mediated through changes in the k_{on} for the reaction, since k_{off} is less dramatically changed over the same pH range.

DISCUSSION

The experiments described above show that there is a significant change in the fluorescence properties of $Fc_{\gamma 1}$, SpG^1 or both proteins when they form a complex. The wavelength of maximum emission from $Fc_{\gamma 1}$ is 330 nm and that from SpG^1 is 338 nm. The difference spectrum obtained between the experimentally observed fluorescence and the spectrum obtained by summating the individual spectra of $Fc_{\gamma 1}$ and SpG^1 has a maximum at 330 nm, suggesting that it is the fluorescence of Trp-48 in SpG^1 that is being quenched.

Lancet et al. [25] found that Protein A from *Staphylococcus aureus* caused a quench of the fluorescence from rabbit Fc on binding but caused no change in fluorescence on interaction with human Fc. Fc from rabbit has one additional tryptophan residue (Trp-404) compared with human Fc, situated on the C_H2–C_H3 interface, the proposed binding site for Protein A [22]. As Protein A is devoid of Trp residues and quenching of fluorescence using Protein A was only observed in the presence of rabbit Fc, Lancet et al. [25] concluded that the quenched Trp residue was Trp-404. Since the IgG-binding domains of Protein A and SpG compete for the same regions on the Fc, the absence of Trp-404 in human Fc and the absence of an effect of Protein A on the fluorescence spectrum of human Fc suggests that the quenching observed in Figure 3 may be attributed to Trp-48 of SpG^1. This conclusion is supported by the observed NMR shift for this residue on complex formation, indicative of its involvement in binding [23].

The rapid change of fluorescence intensity which occurs after mixing SpG^1 and $Fc_{\gamma 1}$ together has been used to determine the kinetic constants k_{on} and k_{off} for the binding and dissociation reactions. The dependence of the rate of change of fluorescence on the concentration of reactant shows that the binding process is rate-limited by the concentration of the reactants, at least up to the maximum concentration of SpG^1 used in the experiments (50 μM; results not shown), which gave a binding $k_{app.}$ of 90 s^{-1}. The higher K_d value (2 μM) observed in pre-equilibrium studies suggests that a change in affinity occurs after initial complex formation (or, rather, after the change in fluorescence intensity has occurred), possibly due to a conformational change in either protein which results in a change in either k_{on} (increase) or k_{off} (decrease) or both (K_d = 300 nM).

One possible model is shown in Scheme 1, where a conformational change occurs in SpG^1 after binding to the $Fc_{\gamma 1}$. At the concentrations of SpG^1 used, $k_{app.}$ for the change in fluorescence properties of the protein(s) (probably SpG^1) upon binding must describe k_1 in Scheme 1 since, like $k_{app.}$, k_1 is the only rate that is dependent upon the concentration of SpG^1. Unless the rate of the putative conformational change in the forward direction (k_2) following initial binding is greater than 90 s^{-1} (given at the highest concentration of SpG^1 used; results not shown), the change in fluorescence intensity also occurs at the reaction described by k_1 and k_{-1} (the equilibrium between free and complexed proteins).

The value of 3 s^{-1} determined at 25 °C for the rate of dissociation of SpG^1 from $SpG^1 \cdot Fc_{\gamma 1}$ at pH 6.0 most probably describes k_{-2}, since the apparent rate of increase of fluorescence corresponding to the overall dissociation process is independent of the free concentration of SpG^1 and is therefore probably rate-limited by a preceding conformational change [34]. In order for the complex at equilibrium to have a dissociation constant 6-fold lower than that given in pre-equilibrium studies, the ratio of k_2 to k_{-1} should be 6. However, it is clear that this structural change is relatively small, and the difference in the values of the two dissociation constants determined before and after the attainment of equilibrium suggests that the change in binding energy is approx. 4 kJ/mol.

$$Fc + SpG^1 \underset{k_{-1}}{\overset{k_1}{=}} Fc \cdot SpG^1 \underset{k_{-2}}{\overset{k_2}{=}} Fc \cdot SpG^{1*}$$

Scheme 1 Binding of SpG^1 to $Fc_{\gamma 1}$

k_1, k_2, k_{-1} and k_{-2} are the rates of the reactions indicated. See the text for details.

The possibility of such a conformational change taking place upon complex formation, i.e. a change to which Trp-48 is sensitive, is consistent with other studies. When hydrogen–deuterium exchange rates in the free and IgG-bound single domain of Protein G were compared [16], it was found that exchange rates of amide protons distant from the putative contact site were affected. It was concluded that the conformational dynamics of the domain are clearly altered by IgG binding. Furthermore, no protection of the hydrogen–deuterium exchange could be detected for Trp-48 (Trp-43 in the numbering system of Orban et al. [16]) because it was too fast even in the complex, implying mobility and/or solvent accessibility. In the NMR study of SpG–IgG Fc complex formation [23], it was noted that some of the observed chemical shifts may result from conformational changes caused by complex formation rather than (or in addition to) direct protein–protein contact. One of the largest shifts was recorded for the Nϵ proton of the Trp side-chain. The direct involvement of this residue, together with others from the β3 strand, is also implicated, however, by peptide binding studies [24], but the lack of any additional binding affinity afforded when residues of the α-helix were also included in a longer peptide may imply that at least some of the chemical shifts observed by Gronenborn and Clore [23] for the α-helical residues are due to indirect conformational effects. In addition, when the various X-ray- and NMR-derived structures for single domains of SpG are compared [18], including a direct comparison of chemically identical domains in solution and in the crystal [19], the principal differences are detected in the relative orientations of the α-helix and the β sheet. It appears that such conformational differences can readily be accommodated by the structure, despite the otherwise very compact core of the domain [19]. Thus a conformational change to which Trp-48 might be sensitive, when SpG^1 binds to IgG, is certainly possible.

The rate constant k_{off} is only slightly affected by pH, changing from approx. 2 s^{-1} at pH 4 to 1 s^{-1} at pH 8.0 (both determined at 13 °C), suggesting that charged residues are not involved in the dissociation process. In contrast, k_{on} is more sensitive to pH, the rate changing from 0.1×10^6 $M^{-1} \cdot s^{-1}$ at pH 4 to 0.6×10^6 $M^{-1} \cdot s^{-1}$ at pH 6.0 and 0.15×10^6 $M^{-1} \cdot s^{-1}$ at pH 8.0. These changes may reflect the ionizations of acidic residues located in the binding peptide studied by Frick et al. [24] (i.e. Asp-41 or -45) and in the deionization of His residues in the Fc [35] respectively.

The saturation curves for SpG^1-W48F showed a degree of quenching equivalent to that seen with the control protein (inositol monophosphatase) and the K_d value could not be calculated. This result lends itself to two possible interpretations; firstly that the Trp is essential for binding and that the lack of quenching is a direct consequence of the loss in affinity; or secondly that the protein is still able to bind Fc but no significant quenching is observed because the Trp giving rise to the fluorescence that is quenched in the titration with wild-type SpG^1 has been removed. Both of these interpretations are reasonable. However, the implication of the role of the Trp side chain in binding by NMR [23] suggests that its removal would result in a reduction of affinity. In fact our competitive ELISA experiments

have clarified the situation and have shown that this mutated protein forms a very weak complex with $Fc_{\gamma 1}$ with a K_d of approx. 92 μM, thus emphasizing the importance of Trp-48 in the binding interactions.

Most methods to date, with the notable exception of titration calorimetry [19], have required the immobilization of either Protein G or the IgG molecule and have only allowed relative K_d values to be calculated. The K_d value obtained here for the single-domain construct SpG^1 (306 nM) is in good agreement with the value obtained by titration calorimetry for the C1 domain of Protein G (333 nM) and confirms the validity of the method described for determining the affinity of these proteins. This method has also proved useful for determining the K_d value for another IgG-binding protein, SpA_B, with $Fc_{\gamma 1}$ which has not previously been determined in solution. The K_d value obtained for a single-Ig-binding domain of Protein A is similar (27 nM) to that obtained previously (40 nM [36]).

In conclusion, by using a combination of equilibrium and pre-equilibrium techniques using Trp-48 as a site-specific spectroscopic reporter, we have detected evidence for a conformational change in the complex formed between SpG^1 and $Fc_{\gamma 1}$.

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